

Antibody Microarrays 380 & 500 User Manual



Clontech

United States/Canada
800.662.2566

Asia Pacific
+1.650.919.7300

Europe
+33.(0)1.3904.6880

Japan
+81.(0)77.543.6116

Clontech Laboratories, Inc.
A Takara Bio Company
1290 Terra Bella Ave.
Mountain View, CA 94043
Technical Support (US)
E-mail: tech@clontech.com
www.clontech.com

Cat. Nos. 631790
631795
631786
631796
631797
631798

PT3648-1(PR123821)

Published February 2011

Table of Contents

I. Introduction	3
II. Protocol Overview & Troubleshooting Guide	4
III. List of Components	6
IV. Additional Materials & Equipment Required	8
V. Important Pre-Protocol Considerations	9
VI. Protein Extraction & Labeling (Large-Scale) Protocol	10
A. Extracting Protein from Crude Tissue	10
B. Extracting Protein from Cultured Cells	11
C. Preparing Protein from Body Fluids	12
D. Labeling Protein with Fluorescent Dye	13
E. Removing Unbound Dye using PD-10 Desalting Columns	14
F. Determining Protein Concentration	16
G. Estimating the Average Number of Coupled Dye Molecules	17
VII. Protein Extraction & Labeling (Small-Scale) Protocol	19
A. Extracting Protein from Crude Tissue	19
B. Extracting Protein from Cultured Cells	20
C. Labeling Protein with Fluorescent Dye	21
D. Removing Unbound Dye using Protein Desalting Spin Columns	22
E. Determining Protein Concentration	24
F. Estimating the Average Number of Coupled Dye Molecules	25
VIII. Antibody Array Incubation Protocol	27
IX. Analysis of Results	31
X. References	34

List of Figures

Figure 1A. The Ab Microarray procedure (Steps 1–3).	4
Figure 1B. The Ab Microarray procedure (Steps 4, 5).	5
Figure 2. The Ab Microarray Analysis Workbook contains four worksheets.	32
Figure 3. The “Import & Analyses” worksheet has three sections: Import, Analyses, and Sorting.	32
Figure 4. The “Sorting” section of the “Import & Analyses” worksheet	33

List of Tables

Table I. Comparison of protein extraction & labeling methods	9
--	---

Customer Service/Ordering

tel: 800.662.2566 (toll-free)

fax: 800.424.1350 (toll-free)

web: www.clontech.com

e-mail: orders@clontech.com

Technical Support

tel: 800.662.2566 (toll-free)

fax: 650.424.1064

e-mail: tech@clontech.com

I. Introduction

The **Ab Microarray 500** enables you to detect a wide variety of proteins (both cytosolic and membrane bound) representing a broad range of biological functions. The **Ab Microarray 380 - Disease Profiling Array** enables you to detect many different proteins that are known to be associated with a variety of conditions such as cancer, metastasis, multiple sclerosis, Parkinson's, Alzheimer's, obesity, transplanted organ rejection, digestive disorders, and more. Examples of the Ab Microarray's utility include the study of expression of apoptosis regulatory proteins (Yamagiwa *et al.*, 2004), as well as the study of expression of proteins relevant to diabetes (Gosmanov *et al.*, 2004). This array allows you to detect differences in protein abundance between two individual samples—cells, whole tissue, or biological fluids. The fluorescence-based procedure, which takes less than a day to complete, lets you detect as little as 20 pg/ml of each protein target. A dual-color detection method is uniquely designed so that inherent variations in dye labeling do not affect the outcome of the experiment. Thus, you can be confident that your side-by-side comparisons reflect the relative abundances of proteins in the sample. Follow-up studies with Western blotting and *in situ* hybridization support these claims (Song *et al.*, 2002).

Our proprietary process ensures that antibodies remain functionally active even after they are covalently immobilized to the glass surface. The arrays are printed on 75 x 25 x 1 mm glass slides, an open platform that is compatible with commercially available microarray scanners. All arrayed antibodies are carefully tested for specificity and sensitivity. Those that display a high degree of cross reactivity are eliminated from the final product. For a complete list of the arrayed antibodies, including Swiss-Prot ID numbers of the target antigens, please visit the Bioinformatics or Online Tools page of our web site at www.clontech.com.

Procedural Overview

The Ab Microarray protocol, outlined in Figures 1A & 1B, is a fluorescence-based procedure in which solid-phase antibody is used to capture fluorescently-labeled antigen. The entire procedure, from sample preparation to array scanning, takes one day to complete.

Measuring protein abundances with Ab Microarrays consists of the following five main steps (see Section II for details and troubleshooting information):

Step 1. Extract protein from cells or whole tissue

Step 2. Label protein with Cy5 and Cy3 dyes

Step 3. Remove unbound dye by gel filtration

Step 4. Incubate labeled protein with Ab Microarrays

Step 5. Scan microarrays to measure bound antigen

II. Protocol Overview & Troubleshooting Guide

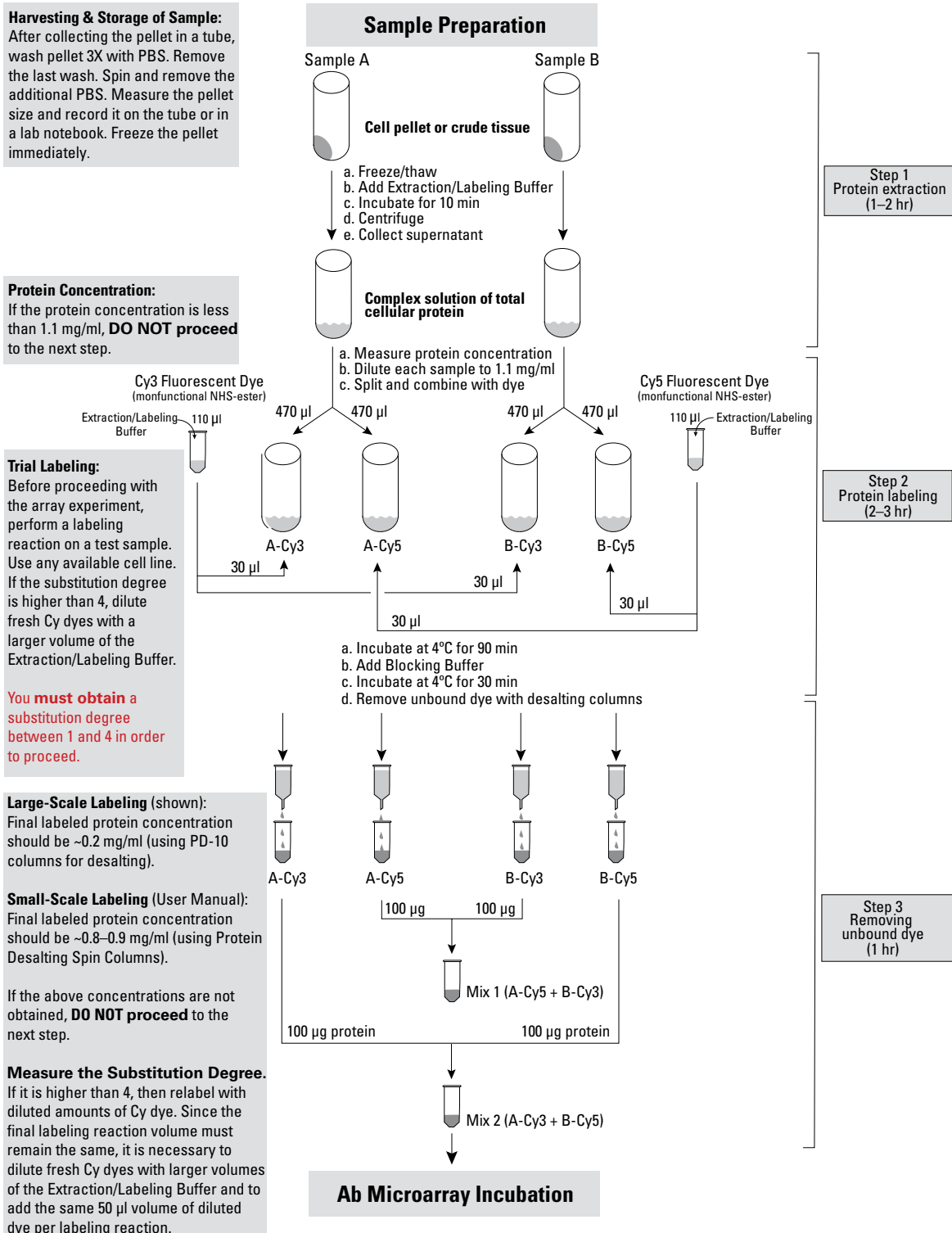


Figure 1A. The Ab Microarray procedure (Steps 1–3). The volumes and quantities given in Steps 1 and 2 correspond to those needed for the Large-Scale Protein Extraction & Labeling procedure. Steps 4 & 5 are shown in Figure 1B.

II. Protocol Overview & Troubleshooting Guide continued

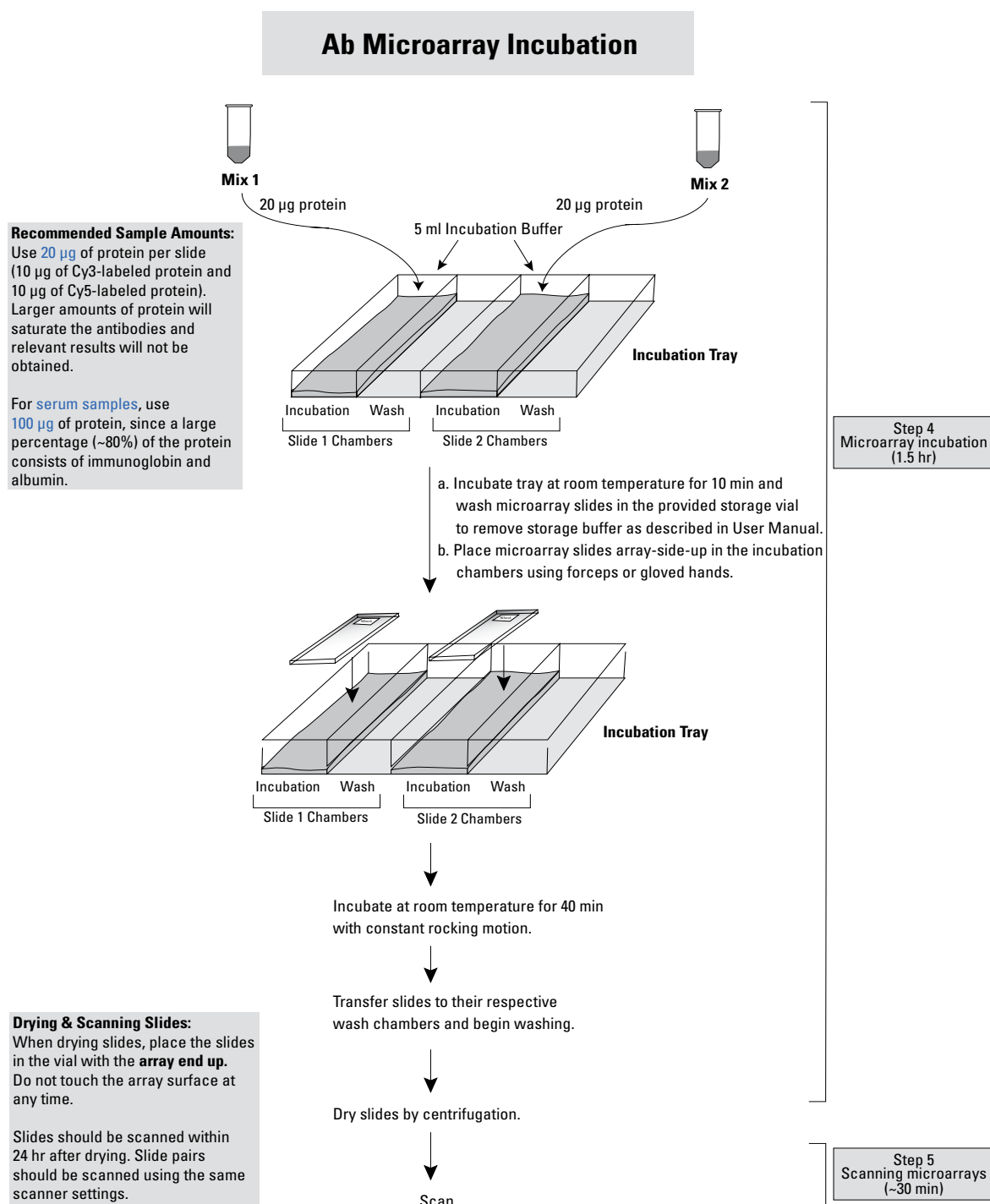


Figure 1B. The Ab Microarray procedure (Steps 4, 5).

III. List of Components

Store Ab Microarray Slides at –20°C.

Store all other components at 4°C.

Ab Microarray Express Buffer Kit* (Cat. No. 631795)

- 2 Incubation Trays
- 20 ml Extraction/Labeling Buffer
- 200 µl Blocking Buffer
- 20 ml 10X Desalting Buffer
- 90 ml Stock Incubation Buffer (2 Bottles)
- 10 ml Background Reducer
- 20 ml Wash Buffer A
- 20 ml Wash Buffer B
- 20 ml Wash Buffer C

- Antibody Microarrays 380 & 500 User Manual (PT3648-1)
- Ab Microarray Analysis Workbook

A Microsoft Excel 97/98 file used for array data analysis. This workbook must be downloaded from the Bioinformatics or Online Tools page of our web site at www.clontech.com

*The Ab Microarray Express Buffer Kit provides reagents suitable for two Ab Microarray experiments (four slides total) as described in this User Manual.

Ab Array 380 - Disease Profiling Array (Cat. No. 631797)

- 2 Ab Microarray 380 slides
- 1 Storage Vial

Ab Array 380 - Disease Profiling Kit (Cat. No. 631796)

- 4 Ab Microarray 380 slides
- 2 Storage Vials
- 1 Ab Microarray Express Buffer Kit (Cat. No. 631795)

Ab Microarray 500 Kit (Cat. No. 631798)

- 4 Ab Microarray 500 slides
- 2 Storage Vials
- 1 Ab Microarray Express Buffer Kit (Cat. No. 631795)

III. List of Components continued

Ab Microarray 500 Slides (Cat. No. 631790)

- 2 Ab Microarray 500 slides
- 1 Storage Vial

Protein Extraction & Labeling Kit (Cat. No. 631786)

This kit is designed for first time users. Use this kit to optimize the labeling reaction. The goal is to obtain the correct dye/molecule ratio to allow for reliable, reproducible results between experiments.

- 20 ml Extraction/Labeling Buffer
- 20 ml 10X Desalting Buffer
- 100 μ l Blocking Buffer

Visit our Antibody Arrays product page at **www.clontech.com** for a current list of products available for use with our Ab Arrays.

IV. Additional Materials & Equipment Required

The following materials are required but not supplied:

- **Alumina** (Sigma Cat. No. A-2039; for disintegrating tissue samples)
- **BCA Protein Assay Reagent Kit** (Pierce Biotechnology; Cat. No. 23225 or 23227)
Pierce's BCA Protein Assay Reagent Kit has been tested by our scientists and is approved for use with Ab Microarray procedures and reagents.
- **Bovine serum albumin** (BSA; protein standard)
- **0.1 M sodium carbonate buffer** (pH 8.3)
Used in the preparation of body fluids for array analysis
- **Cy5 mono-Reactive Dye Pack** (GE Healthcare, Cat. No. PA25001)
- **Cy3 mono-Reactive Dye Pack** (GE Healthcare, Cat. No. PA23001)
Cy5 and Cy3 are fluorescent dyes that have distinct emission spectra.
- **1.5 ml and 2.0 ml microcentrifuge tubes**
- **15 ml and 50 ml conical centrifuge tubes** (e.g., BD Falcon™ conical centrifuge tubes)
- **Disposable PD-10 Desalting Columns**
(GE Healthcare; 17-0851-01) These columns are recommended for the Large-Scale Protein Extraction & Labeling Protocol.
- **Protein Desalting Spin Columns** (Pierce Biotechnology; Cat. Nos. 89849 or 89862)
These columns are recommended for the Small-Scale Protein Extraction & Labeling Protocol.
- **Mortar & pestle** (for grinding tissue)
- **Rocking platform** (to provide a constant “see-saw” motion during slide incubation and washing)
- **Swinging-bucket centrifuge** (with adaptors for spinning 50 ml tubes)
- **Microcentrifuge**
- **Spectrometer** (capable of measuring absorbance at 552 and 650 nm)
- **Microarray slide scanner**
You may use any scanner that is compatible with 75 x 25 x 1 mm slides and capable of dual-color analysis. The scanner must be capable of measuring Cy5 and Cy3 fluorescent labels.
- **Microsoft® Excel 97/98 or later** (software application)
Used for calculating Internally Normalized Ratios based on fluorescence data from a microarray analysis.

V. Important Pre-Protocol Considerations



A. Handling Ab Microarray Slides

- Wear laboratory gloves whenever handling Ab Microarrays. Alternatively, use tweezers to manipulate slides.
- Always hold slides at the end nearest the affixed data label. (Note: This label includes identifying information for the array.)

B. Orienting Microarrays

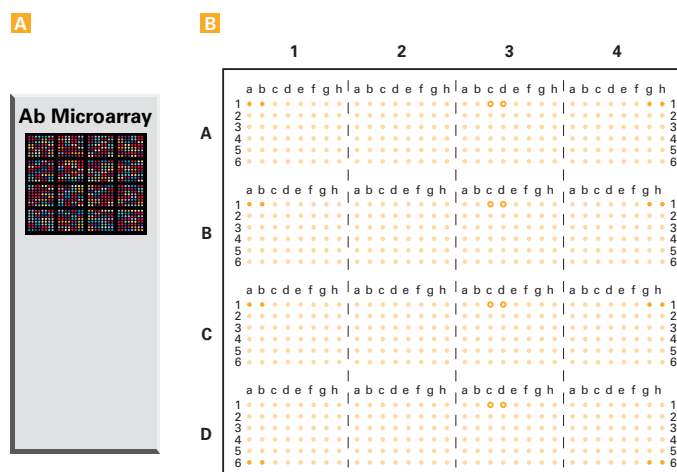


Figure 2. Layout of the Ab Microarray 500. The Ab Microarray 500 contains at least 500 distinct antibodies arrayed in a 32 x 32 grid on a 75 x 25 x 1 mm glass slide (**Panel A**). Each antibody is printed in duplicate. **Panel B**. Darker dots at the corners represent Cy3™/Cy5™-labeled bovine serum albumin (BSA) spots, which serve as orientation markers. The open circles correspond to unlabeled BSA spots, which serve as negative controls. The Ab Microarray 380 has a similar layout to the Ab Microarray 500. The difference is that rows 5 and 6 are not printed in the Ab Microarray 380.

C. Choosing Your Protocol

In completing an Ab Microarray analysis, you have the option of using either a Large-Scale or Small-Scale Protein Extraction & Labeling protocol. The key differences between these two protocols are summarized in Table I.

TABLE I. COMPARISON OF PROTEIN EXTRACTION & LABELING METHODS		
Quantities Needed for or Obtained from Specific Steps ^a	Small-Scale ^b Tissues & Cells	Large-Scale ^b Tissues, Cells & Body Fluids
Quantity of tissue or cells required for protein extraction (Step 1)	15–25 mg	50–200 mg
Expected yield of total protein following protein extraction (Step 1)	~250–500 µg	~2–3 mg
Quantity needed for labeling (Step 2)	~200 µg	~1 mg
^a Diagrammed in Figure 1A		
^b The quantities and volumes given correspond to those needed for a single sample. Recall that for each array analysis, two different samples (referred to as Samples A and B in Figure 1A) are prepared and analyzed.		

Both protocols yield sufficient protein to perform a single antibody microarray analysis. The large-scale method, however, yields more than enough protein for additional downstream analyses performed in conjunction with the antibody microarray procedure. Note, however, that we do not recommend storing labeled protein for long periods because of the potential for protein degradation.

D. Preventing Protein Degradation

DO NOT USE protease inhibitors. The Extraction/Labeling, Blocking, Desalting, Stock Incubation, and Wash Buffers do not contain protease inhibitors because of their potential interference with protein labeling. We obtain excellent results without protease inhibitors, and thus do not recommend their use during protein extraction, labeling, or array detection. We do, however, recommend that once you start the extraction you work quickly and proceed diligently towards the array analysis step.

VI. Protein Extraction & Labeling (Large-Scale) Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING.

Follow the appropriate section below, depending on your starting material: crude tissue (Section A), cultured cells (Section B), or body fluids (Section C).



A. Extracting Protein from Crude Tissue

1. Chill the following items on ice or at 4°C:

- Extraction/Labeling Buffer
- one mortar & pestle
- two 2 ml microcentrifuge tubes
- one 15 ml conical centrifuge tube

2. Perform protein extraction

Frozen tissue

100–200 mg

transfer to a prechilled mortar

Add **alumina**

0.25–0.5 g

to the mortar

Use the pestle to grind the tissue until a paste is formed.

Add prechilled **Extraction/Labeling Buffer**

1–2 ml

to the mortar

Mix the buffer into the paste using the pestle.

Use a micropipette tip to scrape the paste that adheres to the pestle back into the mortar.

Transfer the **extract** to a prechilled 2 ml microcentrifuge tube

Rinse pestle with **Extraction/Labeling Buffer**

1–2 ml

Hold the pestle over the mortar,
rinse the pestle

Combine the **rinse** with the **original extract** in a 2 ml tube

If the volume exceeds the tube's capacity, use a second 2 ml tube

Centrifuge **suspension**

10,000 x g for 30 min

Transfer the supernatant to a prechilled 15 ml conical centrifuge tube

Do not disturb the pellet

Mix **lysate**, by gently inverting the tube

VI. Protein Extraction & Labeling (Large-Scale) Protocol continued

3. Measure protein concentration & dilute sample

Measure protein concentration

Use Pierce's BCA Protein Assay Reagent Kit

Dilute sample with **Extraction/Labeling Buffer**

1.1 mg protein/ml

Final volume must be

≥1 ml

Proceed **immediately** to Section D

If final volume is below 1 ml – STOP Do not proceed to the next step. Repeat the extraction with a fresh sample or carry out small-scale labeling (starting at Section VII.C).



B. Extracting Protein from Cultured Cells

Note: For adherent cells that are 90% confluent, we find that two 150 mm culture plates, when combined, yield ~150 mg of cells. We typically harvest two 150 mm plates for each Sample A and B. Before starting the freeze-thaw procedure, we wash the cells four times with PBS (20 volumes each wash).

1. Cell preparation

Cultured cells

50–150 mg

Centrifuge in a preweighed centrifuge tube

Decant the supernatant

Aspirate the residual liquid

Centrifuge the tube again (for ~2 min)

Aspirate any residual traces of liquid

Weigh the **cell pellet**

Reweigh the tube

Freeze the **cell pellet**

**liquid nitrogen (–196°C)
–80°C freezer**

Place samples in liquid nitrogen or freezer

2. Perform protein extraction

Add **Extraction/Labeling Buffer**

20 µl buffer/ 1 mg cells

Mix thoroughly by vortexing until mixture is homogeneous

Incubate at room temperature

10 min

Constant rotation/mixing

Centrifuge suspension

10,000 x g for 30 min at 4°C

Transfer the **supernatant** to a clean tube

Discard the **pellet**

VI. Protein Extraction & Labeling (Large-Scale) Protocol continued

3. Measure protein concentration & dilute sample

Measure protein concentration

Use Pierce's BCA Protein Assay Reagent Kit

Dilute sample with **Extraction/Labeling Buffer**

1.1 mg protein/ml

Final volume must be

≥1 ml

Proceed **immediately** to Section D

If final volume is below 1 ml – STOP. Do not proceed to the next step. Repeat the extraction with a fresh sample or carry out small-scale labeling (starting at Section VII.C).



C. Preparing Protein from Body Fluids

Note: A preliminary estimate of the protein concentration, followed by a desalting procedure and a second protein assay, is necessary to accurately measure the protein levels in body fluids. Desalting may be performed at room temperature if you work quickly, or else at 4°C.

1. Measure protein concentration & dilute sample

Measure protein concentration

Use Pierce's BCA Protein Assay Reagent Kit

Dilute sample with **0.1 M sodium carbonate buffer (pH 8.3)**

~4 mg protein/ml

2. Perform desalting

Add **0.1 M sodium carbonate buffer (pH 8.3)**

3 x 5 ml

To a PD-10 column (equilibration step)

Load

2.5 ml

Sample onto column

Elute with

3.5 ml

0.1 M sodium carbonate buffer (pH 8.3)

Collect **flowthrough** in a clean 15 ml conical centrifuge tube.

3. Measure protein concentration & dilute sample

Measure protein concentration

Use Pierce's BCA Protein Assay Reagent Kit

Dilute sample with **Extraction/Labeling Buffer**

1.1 mg protein/ml

Final volume must be

≥1 ml

Proceed **immediately** to Section D

If final volume is below 1 ml – STOP. Do not proceed to the next step. Repeat the extraction with a fresh sample or carry out small-scale labeling (starting at Section VII.C).

VI. Protein Extraction & Labeling (Large-Scale) Protocol continued



D. Labeling Protein with Fluorescent Dye

IMPORTANT: Prepare dye solutions, mix dyes with protein samples, and centrifuge mixtures rapidly, all without interruption. After the Cy3 and Cy5 dyes are dissolved in buffer, they must be used immediately. Each dye tube contains sufficient dye to label ~1 mg of total protein. You will need at least 1 ml of protein at a concentration of 1.1 mg/ml to proceed with the fluorescent labeling protocol.

1. Label tubes & prepare dye solutions

Label four **1.5 ml microcentrifuge** tubes:

A-Cy3
A-Cy5
B-Cy3
B-Cy5

Add Extraction/Labeling Buffer	110 μl	To Cy3 dye tube
Vortex dye solution		20 sec
Centrifuge dye solution		10 sec (moderate speed)
Add Extraction/Labeling Buffer	110 μl	To Cy5 dye tube
Vortex dye solution		20 sec
Centrifuge dye solution		10 sec (moderate speed)

2. Mix dyes with protein samples

Add Cy3 solution	30 μl	To tubes A-Cy3 and B-Cy3
Add Cy5 solution	30 μl	To tubes A-Cy5 and B-Cy5
Add Protein Sample A	470 μl	To tubes A-Cy3 and A-Cy5
Add Protein Sample B	470 μl	To tubes B-Cy3 and B-Cy5
Invert each tube 3 times to mix the contents		
Centrifuge protein and dye mixture		10 sec (moderate speed)

VI. Protein Extraction & Labeling (Large-Scale) Protocol continued

3. Incubate dye-sample mixtures

Wrap each tube in foil & **incubate on ice (or at 4°C)**

90 min

Mix by inversion every 20 min or incubate on a rocker at 4°C

Add **Blocking Buffer**

4 µl

To each tube

Wrap each tube in foil & **incubate on ice (or at 4°C)**

30 min

Mix by inversion every 10 min

Proceed **immediately** to Section E.



E. Removing Unbound Dye using PD-10 Desalting Columns

Note: Dye removal using PD-10 desalting columns, manufactured by GE Healthcare (Cat. No. 17-0851-01), may be performed at room temperature if you work quickly, or else at 4°C.

1. Label microfuge tubes & columns, and prepare buffer

Label four **PD-10 Desalting Columns**

A-Cy3

A-Cy5

B-Cy3

B-Cy5

Label four **2 ml microcentrifuge tubes:**

A-Cy3

A-Cy5

B-Cy3

B-Cy5

Prepare **1X Desalting Buffer**

100 ml

Dilute 10X Desalting Buffer with Milli-Q H₂O (in clean plastic bottle)
Adjust the pH to 7.4

VI. Protein Extraction & Labeling (Large-Scale) Protocol continued

2. Perform desalting

Add 1X Desalting Buffer	3 x 5 ml each	To each PD-10 column (equilibration step)
Load the Cy3- and Cy5-labeled protein samples	~500 μl each	Allow protein to pass into column
Add 1X Desalting Buffer	2 ml each	To each PD-10 column Allow buffer to pass into columns
Elute each column by adding 1X Desalting Buffer	2 ml each	To each PD-10 column Collect flowthrough in the prelabeled 2 ml microcentrifuge tubes
Store tubes on ice		

3. Measure protein concentration & dilute sample

Measure protein concentration	Use Pierce's BCA Protein Assay Reagent Kit (see Section F)
-------------------------------	---

VI. Protein Extraction & Labeling (Large-Scale) Protocol continued



F. Determining Protein Concentration

We recommend the **BCA Protein Assay Reagent Kit from Pierce Biotechnology**. It has been tested by our scientists and shown to be compatible with our buffers. Using other BCA reagents (or kits) could lead to errors in protein estimation.

1. Standard curve

Use **bovine serum albumin (BSA)** as your protein standard.

Dilute **BSA** to:

0.02 mg/ml
0.05 mg/ml
0.1 mg/ml
0.2 mg/ml
0.3 mg/ml
0.4 mg/ml
0.5 mg/ml

Use 1 X **Desalting Buffer**

0 mg/ml

As the blank

Measure each sample in triplicate.

2. Subtract contribution of dyes (Cy3 and Cy5 absorb at 562 nm)

Prepare **protein blank**

Substitute BCA reagent with 1X Desalting Buffer
Add an aliquot of your labeled protein

3. Sample plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std. 0 mg/ml	Std. 0.02 mg/ml	Std. 0.05 mg/ml	Std. 0.1 mg/ml	Std. 0.2 mg/ml	Std. 0.3 mg/ml	Std. 0.4 mg/ml	Std. 0.5 mg/ml	Protein blank – Sample A Cy3	Protein blank – Sample A Cy5	Protein blank – Sample B Cy3	Protein blank – Sample B Cy5
B	Std. 0 mg/ml	Std. 0.02 mg/ml	Std. 0.05 mg/ml	Std. 0.1 mg/ml	Std. 0.2 mg/ml	Std. 0.3 mg/ml	Std. 0.4 mg/ml	Std. 0.5 mg/ml	Protein blank – Sample A Cy3	Protein blank – Sample A Cy5	Protein blank – Sample B Cy3	Protein blank – Sample B Cy5
C	Std. 0 mg/ml	Std. 0.02 mg/ml	Std. 0.05 mg/ml	Std. 0.1 mg/ml	Std. 0.2 mg/ml	Std. 0.3 mg/ml	Std. 0.4 mg/ml	Std. 0.5 mg/ml	Protein blank – Sample A Cy3	Protein blank – Sample A Cy5	Protein blank – Sample B Cy3	Protein blank – Sample B Cy5
D	Sample A Cy3	Sample A Cy3	Sample A Cy3	Sample A Cy5	Sample A Cy5	Sample A Cy5	Sample B Cy3	Sample B Cy3	Sample B Cy3	Sample B Cy5	Sample B Cy5	Sample B Cy5
E												
F												
G												
H												

VI. Protein Extraction & Labeling (Large-Scale) Protocol continued

4. Calculate protein concentration

$$\Delta OD_{562} \text{ Sample} = [OD_{562} (\text{protein sample}) - OD_{562} (\text{protein blank})]$$

$$\Delta OD_{562} \text{ Std.} = [OD_{562} (\text{standard}) - OD_{562} (\text{blank} = 0 \text{ mg/ml})]$$

Use the ΔOD_{562} and your standard curve to estimate protein concentration.

Typical concentration of desalted samples ~0.2 mg protein/ml



G. Estimating the Average Number of Coupled Dye Molecules

1. Measure absorbance – using a spectrophotometer

Dilute sample with **1X Desalting Buffer**

1/10 dilution

Prepare blank with **1X Desalting Buffer**

Measure **Cy3 absorbance at 552 nm** – can use 1 cm cuvettes

Blank – 1X Desalting Buffer

Sample A-Cy3 – 1/10 dilution in Desalting Buffer

Sample B-Cy3 – 1/10 dilution in Desalting Buffer

Measure **Cy5 absorbance at 650 nm** – can use 1 cm cuvettes

Blank – 1X Desalting Buffer

Sample A-Cy5 – 1/10 dilution in Desalting Buffer

Sample B-Cy5 – 1/10 dilution in Desalting Buffer

Record **A_{552}** and **A_{650}** for all samples

A_{552} Sample A-Cy3 = absorbance 552 nm of Sample A-Cy3 – absorbance 552 nm of blank

A_{552} Sample B-Cy3 = absorbance 552 nm of Sample B-Cy3 – absorbance 552 nm of blank

A_{650} Sample A-Cy5 = absorbance 650 nm of Sample A-Cy5 – absorbance 650 nm of blank

A_{650} Sample B-Cy5 = absorbance 650 nm of Sample B-Cy5 – absorbance 650 nm of blank

2. General considerations

Assume that the **average molecular weight** of protein is **60,000 Da**

Protein concentration – use the values obtained from the BCA method

Molar extension coefficient (ϵ)

ϵ_{552} of Cy3 = **150,000** $M^{-1} \text{ cm}^{-1}$

ϵ_{650} of Cy5 = **250,000** $M^{-1} \text{ cm}^{-1}$

VI. Protein Extraction & Labeling (Large-Scale) Protocol continued

3. Sample calculation – Sample A-Cy3

Sample data

BCA assay results (protein concentration by BCA) = 0.18 mg/ml

Absorbance results (A_{552}) = 0.9

Sample calculation

$$\begin{aligned}\text{Cy3 concentration in sample} &= (A_{552} / \epsilon_{552}) \times 10^6 \\ &= (0.9 / 150,000) \times 10^6 = 6 \mu\text{M}\end{aligned}$$

$$\begin{aligned}\text{Protein concentration in sample} &= [(\text{BCA assay result}) / (\text{average molecular weight})] \times 10^6 \\ &= (0.18/60,000) \times 10^6 = 3 \mu\text{M}\end{aligned}$$

$$\begin{aligned}\text{Dye/protein} &= \text{Cy3 concentration in sample} / \text{protein concentration in sample} \\ &= 6 \mu\text{M} / 3 \mu\text{M} = 2\end{aligned}$$

For best results, the dye/protein ratio should be in the range of 2–4.

When this ratio is significantly greater (e.g., >6), the label may begin to interfere with antigen-antibody binding.

4. Sample calculation – Sample A-Cy5

Sample data

BCA assay results (protein concentration by BCA) = 0.18 mg/ml

Absorbance results (A_{650}) = 1.5

Sample calculation

$$\begin{aligned}\text{Cy5 concentration in sample} &= (A_{650} / \epsilon_{650}) \times 10^6 \\ &= (1.5 / 250,000) \times 10^6 = 6 \mu\text{M}\end{aligned}$$

$$\begin{aligned}\text{Protein concentration in sample} &= [(\text{BCA assay result}) / (\text{average molecular weight})] \times 10^6 \\ &= (0.18/60,000) \times 10^6 = 3 \mu\text{M}\end{aligned}$$

$$\begin{aligned}\text{Dye/protein} &= \text{Cy5 concentration in sample} / \text{protein concentration in sample} \\ &= 6 \mu\text{M} / 3 \mu\text{M} = 2\end{aligned}$$

For best results, the dye/protein ratio should be in the range of 2–4.

When this ratio is significantly greater (e.g., >6), the label may begin to interfere with antigen-antibody binding.

VII. Protein Extraction & Labeling (Small-Scale) Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING.

Follow the appropriate section below, depending on your starting material: crude tissue (Section A) or cultured cells (Section B).



A. Extracting Protein from Crude Tissue

1. Chill the following items on ice or at 4°C:

- Extraction/Labeling Buffer
- one small mortar & pestle
- two 1.5 ml microcentrifuge tubes

2. Perform protein extraction

Frozen tissue

15–25 mg

transfer to a prechilled mortar

Add **alumina**

2–5 mg

to the mortar

Use the pestle to grind the tissue until a paste is formed.

Add prechilled **Extraction/Labeling Buffer**

100–200 µl

to the mortar

Mix the buffer into the paste using the pestle.

Use a micropipette tip to scrape the paste that adheres to the pestle back into the mortar.

Transfer the **extract** to a prechilled 1.5 ml microcentrifuge tube

Rinse pestle with **Extraction/Labeling Buffer**

100–200 µl

Hold the pestle over the mortar,
rinse the pestle

Combine the **rinse** with the **original extract** in a 1.5 ml tube

Centrifuge **suspension**

10,000 x g for 30 min at 4°C

Transfer the supernatant to a prechilled 1.5 ml microcentrifuge tube

Do not disturb the pellet

Mix **lysate**, by gently inverting the tube

VII. Protein Extraction & Labeling (Small-Scale) Protocol continued

3. Measure protein concentration & dilute sample

Measure protein concentration

Use Pierce's BCA Protein Assay
Reagent Kit

Dilute sample with **Extraction/Labeling Buffer**

1.1 mg protein/ml

Final volume must be

≥200 μ l

Proceed **immediately** to Section C

If final volume is below 200 μ l – STOP. Do not proceed to the next step. Repeat the extraction with a fresh sample and use more starting material.



B. Extracting Protein from Cultured Cells

Note: For adherent cells that are 90% confluent, we find that one 100 mm culture plate yields ~17 mg of cells. Before starting the freeze-thaw procedure, we wash the cells four times with PBS (20 volumes each wash).

1. Cell preparation

Cultured cells

15–25 mg

Centrifuge in a preweighed
microcentrifuge tube

Decant the supernatant

Aspirate the residual liquid

Centrifuge the tube again (for ~2 min)

Aspirate any residual traces of liquid

Weigh the **cell pellet**

Reweigh the tube

Freeze the **cell pellet**

**liquid nitrogen (–196°C)
–80°C freezer**

Place samples in liquid nitrogen
or freezer

2. Perform protein extraction

Add **Extraction/Labeling Buffer**

20 μ l buffer/ 1 mg cells

Mix thoroughly by vortexing
until mixture is homogeneous

Incubate at room temperature

10 min

Constant rotation/mixing

Centrifuge suspension

10,000 x g for 30 min at 4°C

Transfer the **supernatant** to a clean tube

Place tube on ice

Discard the **pellet**

VII. Protein Extraction & Labeling (Small-Scale) Protocol continued

3. Measure protein concentration & dilute sample

Measure protein concentration

Use Pierce's BCA Protein Assay
Reagent Kit

Dilute sample with **Extraction/Labeling Buffer**

1.1 mg protein/ml

Final volume must be

≥200 μ l

Proceed **immediately** to Section C

If final volume is below 200 μ l – STOP. Do not proceed to the next step. Repeat the extraction with a fresh sample and use more starting material.



C. Labeling Protein with Fluorescent Dye

IMPORTANT: Prepare dye solutions, mix dyes with protein samples, and centrifuge mixtures rapidly, all without interruption. After the Cy3 and Cy5 dyes are dissolved in buffer, they must be used immediately. Each dye tube contains sufficient dye to label ~1 mg of total protein.

1. Label tubes & prepare dye solutions

Label four **1.5 ml microcentrifuge** tubes:

A-Cy3

A-Cy5

B-Cy3

B-Cy5

Add **Extraction/Labeling Buffer**

110 μ l

To **Cy3** dye tube

Vortex **dye solution**

20 sec

Centrifuge **dye solution**

10 sec (moderate speed)

Add **Extraction/Labeling Buffer**

110 μ l

To **Cy5** dye tube

Vortex **dye solution**

20 sec

Centrifuge **dye solution**

10 sec (moderate speed)

2. Mix dyes with protein samples

Add **Cy3** solution

6 μ l

To tubes **A-Cy3** and **B-Cy3**

Add **Cy5** solution

6 μ l

To tubes **A-Cy5** and **B-Cy5**

VII. Protein Extraction & Labeling (Small-Scale) Protocol continued

Add **Protein Sample A** **94 μ l** To tubes **A-Cy3** and **A-Cy5**

Add **Protein Sample B** **94 μ l** To tubes **B-Cy3** and **B-Cy5**

Carefully pipette up and down several times to mix the contents

Centrifuge **protein and dye mixture** **10 sec (moderate speed)**

3. Incubate dye-sample mixtures

Wrap each tube in foil & **incubate on ice (or at 4°C)**

90 min Mix by inversion every 20 min

Add **Blocking Buffer**

4 μ l To each tube

Wrap each tube in foil & **incubate on ice (or at 4°C)**

30 min Mix by inversion every 10 min

Proceed **immediately** to Section D.



D. Removing Unbound Dye using Protein Desalting Spin Columns

Note: Dye removal using Protein Desalting Spin Columns, which are manufactured by Pierce Biotechnology, Inc. (Cat. No. 89849 or 89862), may be performed at room temperature if you work quickly, or else at 4°C.

1. Label microfuge tubes & columns, and prepare buffer

Label four **Protein Desalting Spin Columns**

A-Cy3
A-Cy5
B-Cy3
B-Cy5

Label four **2 ml microcentrifuge tubes:**

A-Cy3
A-Cy5
B-Cy3
B-Cy5

Prepare **1X Desalting Buffer**

5 ml

Dilute 10X Desalting Buffer with Milli-Q H₂O (in clean plastic bottle).
Adjust the pH to 7.4

VII. Protein Extraction & Labeling (Small-Scale) Protocol continued

2. Prepare desalting columns

- Spin each column to remove storage buffer 1,500 x g for 2 min
- Add **1X Desalting Buffer** 2 x 400 μ l 1,500 x g for 2 min
(after each buffer addition)
- to each column & spin
- Discard flowthrough after each spin.
- Attach prelabeled microfuge tubes to the corresponding columns.
-

3. Perform desalting

- Load the **Cy3- & Cy5-labeled protein samples** ~100 μ l each Allow samples to pass into columns
- Centrifuge to elute desalted protein 1,500 x g for 2 min
- Detach and cap microfuge tubes Store tubes on ice.
-

4. Measure protein concentration & dilute sample

- Measure protein concentration Use Pierce's BCA Protein Assay
Reagent Kit (see **Section E**)

VII. Protein Extraction & Labeling (Small-Scale) Protocol continued



E. Determining Protein Concentration

We recommend the **BCA Protein Assay Reagent Kit from Pierce Biotechnology**. It has been tested by our scientists and shown to be compatible with our buffers. Using other BCA reagents (or kits) could lead to errors in protein estimation.

1. Standard curve

Use **bovine serum albumin (BSA)** as your protein standard.

Dilute **BSA** to:

0.02 mg/ml
0.05 mg/ml
0.1 mg/ml
0.2 mg/ml
0.3 mg/ml
0.4 mg/ml
0.5 mg/ml

Use 1 X **Desalting Buffer**

0 mg/ml

As the blank

Measure each sample in triplicate.

2. Subtract contribution of dyes (Cy3 and Cy5 absorb at 562 nm)

Prepare **protein blank**

Substitute BCA reagent with 1X Desalting Buffer
Add an aliquot of your labeled protein

3. Sample plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std. 0 mg/ml	Std. 0.02 mg/ml	Std. 0.05 mg/ml	Std. 0.1 mg/ml	Std. 0.2 mg/ml	Std. 0.3 mg/ml	Std. 0.4 mg/ml	Std. 0.5 mg/ml	Protein blank – Sample A Cy3	Protein blank – Sample A Cy5	Protein blank – Sample B Cy3	Protein blank – Sample B Cy5
B	Std. 0 mg/ml	Std. 0.02 mg/ml	Std. 0.05 mg/ml	Std. 0.1 mg/ml	Std. 0.2 mg/ml	Std. 0.3 mg/ml	Std. 0.4 mg/ml	Std. 0.5 mg/ml	Protein blank – Sample A Cy3	Protein blank – Sample A Cy5	Protein blank – Sample B Cy3	Protein blank – Sample B Cy5
C	Std. 0 mg/ml	Std. 0.02 mg/ml	Std. 0.05 mg/ml	Std. 0.1 mg/ml	Std. 0.2 mg/ml	Std. 0.3 mg/ml	Std. 0.4 mg/ml	Std. 0.5 mg/ml	Protein blank – Sample A Cy3	Protein blank – Sample A Cy5	Protein blank – Sample B Cy3	Protein blank – Sample B Cy5
D	Sample A Cy3	Sample A Cy3	Sample A Cy3	Sample A Cy5	Sample A Cy5	Sample A Cy5	Sample B Cy3	Sample B Cy3	Sample B Cy3	Sample B Cy5	Sample B Cy5	Sample B Cy5
E												
F												
G												
H												

VII. Protein Extraction & Labeling (Small-Scale) Protocol continued

4. Calculate protein concentration

$$\Delta OD_{562} \text{ Sample} = [OD_{562} (\text{protein sample}) - OD_{562} (\text{protein blank})]$$

$$\Delta OD_{562} \text{ Std.} = [OD_{562} (\text{standard}) - OD_{562} (\text{blank} = 0 \text{ mg/ml})]$$

Use the ΔOD_{562} and your standard curve to estimate protein concentration.

Typical concentration of desalted samples ~0.8 mg protein/ml



F. Estimating the Average Number of Coupled Dye Molecules

1. Measure absorbance – using a spectrophotometer

Dilute sample with **1X Desalting Buffer**

1/10 dilution

Prepare blank with **1X Desalting Buffer**

Measure **Cy3 absorbance at 552 nm** – can use 1 cm cuvettes

Blank – 1X Desalting Buffer

Sample A-Cy3 – 1/10 dilution in Desalting Buffer

Sample B-Cy3 – 1/10 dilution in Desalting Buffer

Measure **Cy5 absorbance at 650 nm** – can use 1 cm cuvettes

Blank – 1X Desalting Buffer

Sample A-Cy5 – 1/10 dilution in Desalting Buffer

Sample B-Cy5 – 1/10 dilution in Desalting Buffer

Record **A_{552} and A_{650}** for all samples

A_{552} Sample A-Cy3 = absorbance 552 nm of Sample A-Cy3 – absorbance 552 nm of blank

A_{552} Sample B-Cy3 = absorbance 552 nm of Sample B-Cy3 – absorbance 552 nm of blank

A_{650} Sample A-Cy5 = absorbance 650 nm of Sample A-Cy5 – absorbance 650 nm of blank

A_{650} Sample B-Cy5 = absorbance 650 nm of Sample B-Cy5 – absorbance 650 nm of blank

2. General considerations

Assume that the **average molecular weight** of protein is **60,000 Da**

Protein concentration – use the values obtained from the BCA method

Molar extension coefficient (ϵ)

ϵ_{552} of Cy3 = **150,000** $M^{-1} \text{ cm}^{-1}$

ϵ_{650} of Cy5 = **250,000** $M^{-1} \text{ cm}^{-1}$

VII. Protein Extraction & Labeling (Small-Scale) Protocol continued

3. Sample calculation – Sample A-Cy3

Sample data

BCA assay results (protein concentration by BCA) = 0.18 mg/ml

Absorbance results (A_{552}) = 0.9

Sample calculation

$$\begin{aligned}\text{Cy3 concentration in sample} &= (A_{552} / \epsilon_{552}) \times 10^6 \\ &= (0.9 / 150,000) \times 10^6 = 6 \mu\text{M}\end{aligned}$$

$$\begin{aligned}\text{Protein concentration in sample} &= [(\text{BCA assay result}) / (\text{average molecular weight})] \times 10^6 \\ &= (0.18/60,000) \times 10^6 = 3 \mu\text{M}\end{aligned}$$

$$\begin{aligned}\text{Dye/protein} &= \text{Cy3 concentration in sample} / \text{protein concentration in sample} \\ &= 6 \mu\text{M} / 3 \mu\text{M} = 2\end{aligned}$$

For best results, the dye/protein ratio should be in the range of 2–4.

When this ratio is significantly greater (e.g., >6), the label may begin to interfere with antigen-antibody binding.

4. Sample calculation – Sample A-Cy5

Sample data

BCA assay results (protein concentration by BCA) = 0.18 mg/ml

Absorbance results (A_{650}) = 1.5

Sample calculation

$$\begin{aligned}\text{Cy5 concentration in sample} &= (A_{650} / \epsilon_{650}) \times 10^6 \\ &= (0.9 / 150,000) \times 10^6 = 6 \mu\text{M}\end{aligned}$$

$$\begin{aligned}\text{Protein concentration in sample} &= [(\text{BCA assay result}) / (\text{average molecular weight})] \times 10^6 \\ &= (0.18/60,000) \times 10^6 = 3 \mu\text{M}\end{aligned}$$

$$\begin{aligned}\text{Dye/protein} &= \text{Cy5 concentration in sample} / \text{protein concentration in sample} \\ &= 6 \mu\text{M} / 3 \mu\text{M} = 2\end{aligned}$$

For best results, the dye/protein ratio should be in the range of 2–4.

When this ratio is significantly greater (e.g., >6), the label may begin to interfere with antigen-antibody binding.

VIII. Antibody Array Incubation Protocol

1. Prepare Incubation Buffer and set up Incubation Tray

Prepare **Incubation Buffer** **45 ml** Mix 4.5 ml **Background Reducer** & 40.5 ml **Stock Incubation Buffer** in a clean plastic bottle or tube

Set up **Incubation Tray** and label the four chambers: **“Slide 1 Incubation”, “Slide 1 Wash”, “Slide 2 Incubation”, “Slide 2 Wash”** Use a pen to mark exterior surface

Add Incubation Buffer **5 ml** To each chamber

2. Prepare protein sample mixes

Label two 1.5 ml microfuge tubes: **“Slide 1 Mix”** and **“Slide 2 Mix”**

Slide 1 Mix Preparation:

Protein Sample A-Cy5 **100 µg**
Protein Sample B-Cy3 **100 µg**

Slide 2 Mix Preparation:

Protein Sample A-Cy3 **100 µg**
Protein Sample B-Cy5 **100 µg**

Leftover Samples A and B can be stored at 4°C (short-term storage) or –20°C (long-term storage) for later use in other applications—e.g., Western blotting, but stored protein samples are not recommended for future microarray analyses.

3. Transfer protein sample mixes to Incubation Tray

Transfer **Incubation Buffer** **5 ml** To Slides 1 & 2 Incubation chambers

Add protein from **Slide 1 Mix** **20 µg*** To Slide 1 Incubation chamber

Add protein from **Slide 2 Mix** **20 µg*** To Slide 2 Incubation chamber

*For **samples derived from serum**, use **100 µg** of protein, since a large percentage (~80%) of the protein consists of immunoglobulin and albumin.

Incubate the tray at RT **10 min** With gentle rocking

Prepare Ab Microarray Slides (**Step 4**) while incubating tray with protein sample mixes.

VIII. Antibody Array Incubation Protocol continued



4. Prepare Ab Microarray Slides for incubation

Important: Use gloved hands or tweezers to hold and manipulate the slides. Never touch the array-end of the slide. Instead, always hold the slide at the end nearest the affixed label.

The Ab array slides are supplied in Storage Buffer inside a green-capped Storage Vial. An empty storage vial for drying the slides is also provided. The Storage Buffer contains glycerol and should be disposed of in a properly labeled waste container.

Decant the **Storage Buffer** from
the green-capped **Storage Vial**

Press your gloved finger against
the top of vial to keep the slides
from falling out.

Wash **Ab Microarray Slides** as follows:

Transfer the slides into the dry, clean Storage Vial provided with the slides

Add **Stock Incubation Buffer**

30 ml

To Storage Vial

Cap **Storage Vial** and slowly invert 10 times

Decant **Storage Buffer** while using your gloved finger to keep the slides from falling out.

Add **Stock Incubation Buffer**

20 ml

To Storage Vial

Cap **Storage Vial** and slowly invert 10 times

Stand the vial upright in a rack.

Record each slide's **lot number**.

Designate one slide as **Slide 1** and the other slide as **Slide 2**.

5. Incubate Ab Microarray Slides with protein sample mixes

Important: Place each slide **array-side-up** into the appropriate incubation chamber in the Incubation Tray (see Step 1). The array is printed on the side to which the label is affixed.

Transfer **Slide 1** from the Storage Vial to the **Slide 1 Incubation chamber**

Transfer **Slide 2** from the Storage Vial to the **Slide 2 Incubation chamber**

Incubate the tray at RT

40 min

With gentle rocking

Note: Use a micropipette tip to pry up one end of each slide while you gently rock the Incubation Tray once or twice. This helps exchange liquid on all sides of the slide.

VIII. Antibody Array Incubation Protocol continued



6. Wash Ab Microarray Slides & prepare for scanning

Important: Use gloved hands to hold and manipulate the slides. Do not touch the array surface of the slides. Instead, hold the slides by their edges.

Add **Wash Buffer A** **5 ml** To each wash chamber

Transfer **Slide 1** from the **Slide 1 Incubation** chamber to the **Slide 1 Wash chamber**

Transfer **Slide 2** from the **Slide 2 Incubation** chamber to the **Slide 2 Wash chamber**

Incubate at RT **5 min** With gentle rocking

Remove the buffer from the chambers

Add **Wash Buffer B** **5 ml** To each wash chamber

Incubate at RT **5 min** With gentle rocking

Remove the buffer from the chambers

Add **Wash Buffer C** **5 ml** To each wash chamber

Incubate at RT **5 min** With gentle rocking

Rinse the slides as follows:

Transfer each slide into a **50 ml conical tube** filled with Milli-Q grade water, with the slide label facing downward, then pour off the water completely.

Dry the slides as follows:

Place the slides, array-end-up, in the empty, green-capped Storage Vial provided, & cap it.

Centrifuge the slides for **5 min** **1,000 x g at RT**

Using gloved hands, uncap the vial. While holding your finger over the top of the vial to prevent the slides from falling out, tip the vial slightly to nudge the slides near the rim of the vial. When the slides protrude by ~2 cm, remove the slides one-by-one.

The slides are now ready for scanning (see Step 7).

VIII. Antibody Array Incubation Protocol continued

7. Scan the Ab Microarray Slides with a Microarray Scanner

Important: If you need to postpone the scanning, keep the slides in a dry chamber and protect them from light until you are ready to scan.

Scanning Arrays:

- Slides should be scanned ≤ 24 hours after drying.
- We routinely scan the arrays with an Axon GenePix 4000B scanner using the following settings:
 - 635 nm (Cy5 channel): PMT = 670 V; power = 33%
 - 532 nm (Cy3 channel): PMT = 550 V; power = 33%.
- The bovine serum albumin control spots can be used as a guide for setting the scanner. These control spots should generally give a fluorescent signal of 2,500–30,000 fluorescence units (FU). If the control spots are $>50,000$ FU this is an indication that the scanner settings are too high; the slides should be rescanned using lower power settings. If you are using a scanner other than the GenePix 4000B instrument, adjust the laser power and PMT (if possible) to obtain a signal within the range of 2,500–9,000 FU for the control spots.
- Some scanners (such as the PerkinElmer ScanArray instrument) adjust the laser settings based on a pre-scan of the slide; these types of scanners generally calculate the correct settings so adjustments are not normally required. However, it is still useful to check the signal intensities of the control spots as the automatic detection settings based on the pre-scan may be incorrect.

IX. Analysis of Results



A. General Tips for Microarray Data Analysis

In order to use our Ab Microarray Analysis Workbook as described below in Part B, you must first calculate the Cy5/Cy3 fluorescent signal ratios for all coordinates on each array. This calculation can usually be done with your array analysis software (e.g., GenePix Pro). The Cy5/Cy3 values are required to calculate Internally Normalized Ratios (INRs), as described below in Part B.

Antibody Array Data Analysis Features:

- A. Internally normalized results

Internal normalization refers to the sampling method described above (Steps 2–3) in which a portion of each protein sample is labeled with a portion of each fluorophore (see Figure 1). By following our protocol, you obtain an Internally Normalized Ratio (INR) for each antibody antigen pair on the microarray. This sampling method controls for differences in labeling efficiency.

After gel filtration (Step 3), the four samples are combined in equal proportions to form two samples:

- Mix 1, which comprises A-Cy5 and B-Cy3
- Mix 2, which comprises A-Cy3 and B-Cy5

One microarray is incubated with Mix 1; the second microarray is incubated with Mix 2. In this setup, Array 1 measures A-Cy5/B-Cy3 (Ratio 1), while Array 2 measures B-Cy5/A-Cy3 (Ratio 2).

After the slides are scanned, arrange the fluorescence data in our Ab Microarray Analysis Workbook (Microsoft® Excel 97/98), located on the the Bioinformatics or Online Tools page of our web site at www.clontech.com, and obtain an Internally Normalized Ratio for each coordinate on the array by computing $\sqrt{\frac{\text{Ratio 1}}{\text{Ratio 2}}}$.

This value now represents the abundance of an antigen in Sample A relative to that of Sample B. Please see Section B.2 for more details.

- B. Controls

Several bovine serum albumin (BSA) spots are included on all Ab Microarrays. Some of these spots, prelabeled with Cy3 and Cy5, serve as positive controls, and, as discussed in Section V, serve as orientation markers to help you identify the printed area of the microarray. Other BSA spots, not labeled with fluorophore, serve as negative controls. The coordinates of all BSA spots are given on the Certificate of Analysis.

B. Using the Ab Microarray Analysis Workbook to Calculate Internally Normalized Ratios

The Ab Microarray Analysis Workbook is a Microsoft® Excel 97/98 file that converts your fluorescence data into Internally Normalized Ratios (INRs) for each coordinate on the array. As described in the Introduction (Section I), the INR calculated by our workbook is a numerical value that represents the abundance of antigen in Sample A relative to that of Sample B.

To get started:

1. Connect to the Bioinformatics or Online Tools page of our web site at **www.clontech.com** and download a copy of the workbook that corresponds to the Slide Lot Number of your Microarray. The Microarray Slide Lot Number is given on the data label affixed to the glass slide.

NOTE: The Microarray Slide Lot Number differs from that of the assembled Kit. The Kit Lot Number is shown on the Certificate of Analysis and on the labels affixed to Boxes 1 and 2.



IX. Analysis of Results continued

- Launch Microsoft Excel. Then open the Microarray Analysis Workbook.

Upon opening the workbook, you will notice that it contains four worksheets. The names of these sheets appear on tabs at the bottom of the workbook window (Figure 2).



Figure 2. The Ab Microarray Analysis Workbook contains four worksheets.

The “Ab,” “Array,” and “Ab List” worksheets contain array-specific information such as the names and coordinates of antibodies and the Locus Link and SWISS-PROT accession numbers of the corresponding protein targets.

The fourth worksheet, “Import & Analyses,” contains formulas that perform arithmetic operations on the fluorescence data (i.e., Cy5/Cy3 signal ratios) that you paste into the worksheet. Other formulas in this sheet combine the values of these operations to generate an INR for each coordinate on the array. The INR can be represented by the following expression:

$$\text{INR} = \sqrt{\frac{\text{Ratio 1}}{\text{Ratio 2}}}$$

$$\text{where Ratio 1} = \frac{\text{A-Cy5 relative fluorescence units}}{\text{B-Cy3 relative fluorescence units}}$$

$$\text{and Ratio 2} = \frac{\text{B-Cy5 relative fluorescence units}}{\text{A-Cy3 relative fluorescence units}}$$

Note that the Ratio 1 values are obtained from Slide 1. Whereas the Ratio 2 values are obtained from Slide 2.

- Click on the “Import & Analyses” tab to make it the active window.
- Paste the Cy5/Cy3 signal ratios from each array into the appropriate columns of the worksheet (Figure 3). Be sure that your Cy5/Cy3 ratios are listed in the same order as the corresponding Ab-Ag in the worksheet.

When you paste your data into the worksheet, it automatically calculates Ratio 1/Ratio 2 and places these values in the next column, which in Figure 3 is labeled “R/R.”

IMPORT			
Step #1	Paste Slide #1 Cy5/Cy3 Ratio	Paste Slide #2 Cy5/Cy3 Ratio	Ratio of Ratios (Slide#1/Side#2)
	↓	↓	
Ab-Ag	Slide#1	Slide #2	R/R

Figure 3. The “Import & Analyses” worksheet has three sections: Import, Analyses, and Sorting. To use this worksheet, first paste your fluorescence data into the Import section (shown). Be sure the data correspond to the correct antibody-antigen (Ab-Ag) pairs given in the leftmost column. Note: The view shown is that from the Ab Microarray Workbook.

- Choose File>Save As, and save a copy of the workbook under a new name.
- Copy the data in the Ab-Ag, (Average) R/R, and (Average) INR columns.

IX. Analysis of Results continued

- Paste the data into the corresponding columns in the “Sorting” section of the worksheet (Figure 4).

SORTING		
Step #3	Copy the data in columns E, F and G (row 6-517). Paste as values in columns H, I and J from row 6 and sort by Average INR (column J).	
	Ab-Ag	Average R/R Average INR

Figure 4. The “Sorting” section of the “Import & Analyses” worksheet.

- Choose Data>Sort, and sort the data by (Average) INR in ascending or descending order.

Interpretation of Results:

- Average values (e.g., Average R/R and Average INR) are usually considered invalid if they are based on duplicates that differ by more than 30%. INR values are usually considered invalid if they are based on Cy5/Cy3 ratios in which one or more of the antigen signals is(are) less than twice the background signal.
- In theory, an $INR > 1$ indicates that an antigen is more abundant in Sample A than in Sample B. Conversely, an $INR < 1$ indicates that an antigen is less abundant in Sample A than in Sample B. In reality, experimental and assay variabilities must be taken into account when analyzing your results.
- The two slide approach addresses the majority of variability that could be introduced. Although internal normalization improves the quality of your data and addresses potential differences in dye labeling between samples, it is still not advisable to accept any INR value less than or greater than 1.0 as being a valid change. In our experience, INR values that are ≥ 1.3 or ≤ 0.77 indicate valid changes that signify differences in protein abundance. These threshold values were used successfully by Anderson et al. (2003).
- It is important to note that the threshold values of 1.3 and 0.77 are based on an ideal average INR of 1.0. However, an average INR of 1.0 is not normally obtained in actual experiments. Thus, threshold values should be calculated for each set of slides. To calculate the threshold values, it is necessary to calculate the average INR for the experiment. (The average INR can be obtained by averaging the values in column J of the “Import & Analyses” worksheet of the Ab Microarray Workbook.) The average INR should then be multiplied by 1.3 to obtain the upper threshold value and 0.77 to obtain the lower threshold value for that experiment. This practice should be done for every set of slides. For example, if a given experiment generated an average INR of 1.1, the threshold values would then be 1.43 (1.1×1.3) and 0.85 (1.1×0.77). Thus, for this hypothetical experiment a valid change in protein abundance would be any INR value > 1.43 or < 0.85 .
- To validate your results, you may wish to repeat the assay using individual antibodies with a Western blot procedure.

X. References

- Abbott, A. (1999a) A post-genomic challenge: learning to read patterns of protein synthesis. *Nature* **402**:715–720.
- Abbott, A. (1999b) How to spot a protein in a crowd. *Nature* **402**: 715–717.
- Anderson, K., Potter, A., Baban, D. & Davies, K. E. (2003) Protein expression changes in spinal muscular atrophy revealed with a novel antibody array technology. *Brain* **126**: 2052–2064.
- Blattner, F. R., et al. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277**: 1453–1470.
- Bult, C. J., et al. (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* **273**: 1058–1073.
- Dalton, R. & Abbott, A (1999) Can researchers find recipe for proteins and chips? *Nature* **402**: 719–720.
- Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Murakami, Y., Philippsen, P., Tettelin, H. & Oliver, S. G. (1996) Life with 6000 genes. *Science* **274**:546, 563–7.
- Gosmanov, A. R., Umpierrez, G. E., Carabel, A. H., Cuervo, R. & Thomason, D.B. (9 March 2004) Impaired expression and insulin-stimulated phosphorylation of Akt-2 in muscle of obese patients with atypical diabetes. *Am J Physiol Endocrinol Metab.* **287**(1):E8-E15.
- Haab, B. B., Dunham, M. J. & Brown, P. O. (2001) Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. *Genome Biol.* **2**(2):research0004.1–0004.13.
- Hodgkin, J., Plasterk, R. H. & Waterston, R. H. (1995) The nematode *Caenorhabditis elegans* and its genome. *Science* **270**:410–414.
- Lander, E. S., et al. (2001) Initial sequencing and analysis of the human genome. *Nature* **409**:860–921.
- Simpson, R. J. & Dorow, D. S. (2001) Cancer proteomics: from signaling networks to tumor markers. *Trends Biotechnol.* **19**(suppl.):S40–S48.
- Song, Y., McDuffie, E., Sobocinski, G., States, D. & Albassam, M. (2002) Profiling of human endothelial cells, human aortic smooth muscle cells & human macrophages responses to lipopolysaccharide stimulation using protein microarrays. Poster presented at the First Annual Great Lakes Bioinformatics Retreat, Michigan, USA
- de Wildt, R. M., Mundy, C. R., Gorick, B. D. & Tomlinson, I. M. (2000) Antibody arrays for high-throughput screening of antibody-antigen interactions. *Nat. Biotechnol.* **18**:989–994.
- Venter, J. C., et al. (2001) The sequence of the human genome. *Science* **291**:1304–1351.
- Yamagiwa, Y., Marienfeld, C., Meng, F., Holcik, M. & Patel, T. (2004) Translational regulation of X-linked inhibitor of apoptosis protein by interleukin-6: a novel mechanism of tumor cell survival. *Cancer Res.* **64**:1293–1298.
- Zhou, H., Roy, S., Schulman, H. & Natan, M. J. (2001) Solution and chip arrays in protein profiling. *Trends Biotechnol.* **19**(suppl.): S34–S39.

Notes

Notice to Purchaser

Clontech products are to be used for research purposes only. They may not be used for any other purpose, including, but not limited to, use in drugs, *in vitro* diagnostic purposes, therapeutics, or in humans. Clontech products may not be transferred to third parties, resold, modified for resale, or used to manufacture commercial products or to provide a service to third parties without written approval of Clontech Laboratories, Inc.

Milli-Q® is a registered trademark of Millipore Corporation.

Microsoft® is a registered trademark of Microsoft Corporation.

ScanArray® is a registered trademark of PerkinElmer, Inc.

GenePix™ is a trademark of Axon Instruments.

BD Falcon™ is a trademark of Becton, Dickinson and Company.

Clontech, the Clontech logo and all other trademarks are the property of Clontech Laboratories, Inc., unless noted otherwise.
Clontech is a Takara Bio Company. ©2011 Clontech Laboratories, Inc.